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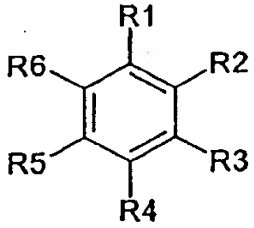

(I)

(57) Abstract

Compounds having formula (I): wherein R^1 is $CH=CH_2$, COOH, CH_2-CH_3 or OH; R^3 is OH, or $-OCH_3$; R^4 is OH; and R^2, R^5 and R^6 are hydrogen, the compound of formula (I) optionally being in the form of a salt when it contains a COOH and/or OH group, may be used as multifunctional preservatives having two or more activities selected from antioxidant, antimicrobial, antibrowning, flavouring, aroma and skin lightener activities.

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PRESERVATIVE COMPOUNDS AND COMPOSITIONS

The present invention relates to compounds from natural biological materials such as plant material which display unexpectedly good preservative properties. The compounds may be derived from precursors present in the biological materials and may be useful, for example, for extending shelf-life.

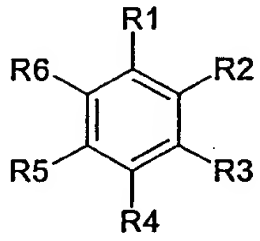
The demand for natural preservative agents is great and increasing in many areas of industry including food, cosmetics, and hygiene. However, this demand is either unmet or met with agents which are unsatisfactory. For example, synthetic chemical preservatives may have adverse environmental effects as they tend to be poorly biodegradable or liberate undesirable compounds such as formaldehyde.

Also, since a combination of preservative activities eg. antimicrobial and antioxidant may be required for a particular application, a combination of preservatives must be used. This may give rise to possibly adverse interactions of two or more individual preservative components or simply increase the cost of providing the overall preservative activity sought, and/or create difficulties in formulating and manufacturing the product(s).

The present invention seeks to reduce or overcome these and other problems of known preservatives.

Ferulic acid is known to have some skin lightening and antioxidant activity. Protocatechuic acid is known to have antioxidant activity but not other beneficial properties.

In a first aspect the invention provides use of a compound having the following formula I:



I

wherein R^1 is $CH=CH_2$, COOH, CH_2-CH_3 or OH;

R^3 is OH, or -OCH_3;

R^4 is OH; and

R^2, R^5 and R^6 are hydrogen,

the compound of formula I optionally being in the form of a salt (including mono-, di- and poly- valent salts) when it contains a COOH and/or OH group,

as a multifunctional preservative having two or more activities selected from antioxidant, antimicrobial, antibrowning, flavouring/aroma and skin lightener activities.

The mutifunctional activity of the compounds means that they can replace two or more compounds having different activities in conventional compositions. This is clearly advantageous.

Preferably, the compound is selected from one or more of vinylguaiacol, ethylguaiacol, vinylcatechol, methoxyhydroquinone and protocatechuic acid. More preferably, the compound is vinylguaiacol, ethylguaiacol, or protocatechuic acid.

Antioxidants have two main uses: to preserve the appearance and composition of products; and to provide a health benefit when 'carried over' into the consumer. Antioxidants are used to prevent the rancidity of a range of fats and oils.

The natural antioxidants used currently, such as tocopherols, are less active, more expensive and also less stable than known synthetic antioxidants, whereas some of the multifunctional natural antioxidants of the invention are more active than synthetic antioxidants. In particular, whereas synthetic antioxidants are very effective in eventually scavenging free radicals, their rate of scavenging is markedly less than many of the compounds of the invention so that the compounds of the invention are particularly useful when the free radicals need to be scavenged quickly to prevent damage, eg, on the skin when used in skin care compositions.

Because of the natural food status of the antioxidants of the invention, they can be used first to preserve a food, beverage, fruit, vegetable, cosmetic, skincare, or personal-care product; so as to extend its shelf life, and then when used the antioxidant is quite consistent with having a positive health effect either, for instance, when ingested in the case of a food or beverage, or absorbed into the skin in the case of cosmetics.

Some of the compounds of the invention have antimicrobial activity. By "antimicrobial" we include the meaning that the compound prevents or inhibits the growth of one or more microorganisms such as bacteria and fungi. This may include the killing of or the prevention of growth of microorganisms and/or a reduction or prevention of the production of microbial metabolities which may have an adverse effect on the composition, structure, appearance, taste, flavour, smell, or safety of the material in which they are disposed.

Food spoilage by micro-organisms occurs in processed foods and fruit and vegetables, both in the field and most importantly post-harvest.

Preferably the compounds of the invention are active against one or more of the following groups of bacteria: Pseudomonads, especially *P.aeruginosa*; *Propionobacter acnes, Pityosporium ovale, Pseudomonas cepacia*; Staphylococci, especially *S. aureus*; *Salmonella*, especially *S.choleraesuis*; Streptococci, especially *S. mutans*; *Escherichia coli*; Bacillus, especially *B. cereus* and *B.subtilis*; Listeria, especially *L. monocytogenes*; and Proteus, especially *P.vulgaris*.

Preferably, the compounds of the invention are active against one or more of the following groups of fungi and yeast: Candida and Aspergillus, especially *C. albicans, A. niger* and *A. flavus*; and Penicillium especially *P.chrysogenum*.

Vinylcatechol is particularly preferred for use as an antimicrobial against *P. aeroginosa* and/or *P. ovale*. Vinylguaiacol is particularly preferred for use as an antimicrobial against *P. ovale*.

Currently there are no generally applicable (broad spectrum), active and cost-effective natural antimicrobial agents.

Few of the commercially available antimicrobials are very effective against the really troublesome strains, such as *P. aeruginosa*, that create most of the problems. One example is Bronopol but it is a synthetic agent ie, not natural, and has no other useful properties such as antioxidant activity.

The antimicrobial agents provided by the invention are particularly valuable because they may display a combination of activities eg low minimum inhibitory concentration allied with broad spectrum activity and/or effectiveness over broad pH and temperature ranges.

The compounds of the invention may also have antibrowning activity. By "antibrowning" we include the meaning that the compound prevents or reduces undesirable colour formation in a food, beverage, fruit and vegetable products. This effect is achieved by inhibiting polyphenol oxidase enzymes such as, for example, tyrosinase and/or laccase, that act on molecules such as chlorogenic acid which are present in the food or beverage.

Preferred compounds for laccase inhibition include vinylguaiacol and ethylguaiacol.

The compounds of the invention may also have activity as a skin lightener. By "skin lightener" we include the meaning that the compound inhibits the enzymes (such as tyrosinase) which are associated with the formation of melanin.

Skilled persons will appreciate that the antibrowning and skin lightening activities of the compounds of the invention are linked in that, without intending to be bound in any way by scientific theory, they appear to work by inhibiting enzymes associated with coloured material formation. For example, the compounds inhibit polyphenol oxidase (tyrosinase and laccase) enzymes which catalyse formation of brown-black pigments (melanoids) formed by oxidative polymerization of plant phenols such as chlorogenic acid in plant tissue. Similarly, the compounds of the invention inhibit tyrosinase enzyme in the skin to prevent melanin formation from precursor materials present in skin cells, such as tyrosine.

The compounds of the invention can be used in a wide range of applications because of their versatility. For instance, at alkaline, neutral and acidic pHs as low as pH 2.8 useful antimicrobial activity has been observed. The broad spectrum activities include antimicrobial activity against a range of microorganisms both in personal-care and food uses, for instance, *P. aeruginosa, S.aureus, Salmonella* sps. and *S. mutans*.

A particularly advantageous feature of the compounds of the invention is that they possess two or more different preservative activities ie, they have multiple preservative functionality eg, antioxidant plus antimicrobial, or antioxidant plus antibrowning or antioxidant plus skin lightener or antioxidant plus flavour and/or aroma, etc (see Table 15). Several of the

compounds, particularly those bearing acidic groups, also have acidulant activity. Hence, a single compound of the invention may replace a plurality of preservatives in known compositions.

Without wishing to be bound by any theories, we think that the antimicrobial and antioxidant activities of the compounds of the invention require different mechanisms of action which can operate simultaneously. This allows them to achieve the multiple preservative functionality afforded by the present invention.

A particular advantage of the compounds of the invention is that they display solubility in both oil and water. Most deterioration occurs at interfaces, for example in multiphase foods, at oil-water or air interfaces. Hence, it is advantageous if the preservative material has appreciable solubility in both water and oil so that microbial growth and/or oxidation can be controlled in both phases.

A still further advantage of the compounds of the invention is that they possess a pleasant taste and/or aroma and this may avoid the need for the separate addition of a flavour and/or perfume in various compositions and formulations. This may also make the compounds suitable for so called "deofragrances" ie, aroma chemicals with deodorant activity to mask malodours and antimicrobial activity to prevent malodour formulation.

Compounds such as vinylguaiacol possess both a pleasant aroma and antimicrobial activity, making them especially suitable for use in deodorant formulations.

Vinylguaiacol has a fresh camphoraceous/herbal/medicated character bringing to mind some pharmaceutical cough preparations. Thus, it conveys hygiene, cleanliness and well being.

The compounds can also be used as preservatives in agricultural compositions (eg, pesticides or fungicides) or as a source of "self-preserving" dietary fibre.

Preferably, the multifunctional preservative compound is for use as an antioxidant and antimicrobial and is selected from vinylcatechol, vinylguaiacol or ethylguaiacol.

Preferably, the multifunctional preservative compound is for use as antioxidant and antibrowning agent and is vinylguaiacol.

Preferably the multifunctional preservative compound is vinylguaiacol for use as an antioxidant, antimicrobial or for antibrowning and/or skin lightening.

Preferably, the multifunctional preservative compound is protocatechuic acid or vinylguaiacol for use as an antioxidant and skin lightener.

Preferably, the multifunctional preservative compound is protocatechuic acid for use as an antioxidant and an acidulant.

Preferably, the multifunctional preservative compound is vinylguaiacol or ethylguaiacol for flavour/aroma use plus use as an antimicrobial, antioxidant or skin lightener.

Preferably, the multifunctional preservative is vinylguaiacol and/or ethylguaiacol for use as a flavour and/or aroma agent and an antioxidant and/or antimicrobial agent.

The compounds may be prepared by a bioprocess which comprises treating a substrate with one or more microorganisms selected from *Rhodotorula*, *Saccharomyces* (eg, *S.cerevisiae*), *Paecilomyces*, *Candida*, *Aspergillus* and *Paenibacillus*; wherein the substrate is selected from ferulic acid or caffeic acid.

An advantageous feature of such bioprocesses is that they are natural, that is, they involve biological, especially enzymatic, processes and the molecules are readily biodegradable because they occur in nature and, indeed, are already present in the human food supply from vegetable, food and beverage sources.

Skilled persons will appreciate that the bioprocesses are not limited to the specific examples, but include micro-organisms and/or enzymic and/or cell free extracts and/or genetically engineered cells or enzymes therefrom which exhibit a suitable enzymic activity.

The micro-organism or enzyme or cell-free extract derived therefrom may produce the desired product efficiently and in high yields. This may be quantified in terms of: the rate of production of the product ($gl^{-1}day^{-1}$); the concentration of the product that accumulates (gl^{-1}); the yield of the product obtained from the substrate (g of product per g of substrate or %

M yield); and the absence of side products which is reflected in the purity of the isolated product (% purity).

The strains may exhibit tolerance to high concentrations of both the substrate and the product, for example at least $1gl^{-1}$, preferably in the range of 1 to $40gl^{-1}$, more preferably in the range of 5 to $40gl^{-1}$. The strains may also exhibit high metabolic selectivity for the production of the required products, for example the products may be produced in at least 75% reaction molar yield and at least 50% recovered molar yield, and they have the ability to produce the products as non-growing cells so that, for example, expensive nutrients do not have to be supplied and expensive sterile fermentation equipment does not have to be used.

In particular, the criteria for establishing suitability of the micro-organism or enzyme or cell-free extract for use in the methods of the invention are as follows:

The micro-organism or enzyme or cell-free extract derived therefrom may produce at least 1g of the desired product per litre of reaction fluid and/or at least 50% molar yield of the desired product from the substrate (eg ferulic acid or caffeic acid) at a concentration of $>0.5gl^{-1}$. The desired product may have a purity of at least 90% as determined by positive characterisation of the product by *ab initio* analytical methods such as NMR.

The micro-organism or enzyme or cell-free extract may be capable of being used repeatedly, in two phase reaction systems, as immobilised

cells, as disrupted cells, and is capable of reacting with impure substrates, especially plant extracts, if required.

Preferably, the bioprocess includes a biphasic reaction mixture. More preferably, the biphasic reaction mixture includes an aqueous phase, such as water, and a water immiscible phase (eg, an organic phase) such as vegetable oil, for example miglyol. The water immiscible phase acts as a product 'sink' in which the desired product formed from the substrate accumulates. This prevents accumulation of the product in the aqueous phase to levels which may inhibit or terminate the enzymatic reaction. This results in increased product yields compared to when the bioprocess is performed using a monophasic reaction mixture.

The product should be produced over a reasonably short period of time eg 1 to 3 days or less. Preferably, the test microorganism is isolated using the soil isolation protocol described hereinafter.

Preferably, one or more of the compounds of the invention are provided in the form of an extract from a plant material. Suitable extracts from plant materials include, for example, a maize extract containing vinylguaiacol and an onion skin extract containing protocatechuic acid.

The invention also provides, in another aspect, compositions comprising a compound having a formula I as defined previously in combination with an additive which has at least one of the same activities as the compound.

Preferably the additive is one of the commonly used antioxidants or antimicrobial agents. For instance, the antioxidant is selected from

butyrated hydroxyanisole, butyrated hydroxytoluene, tertiary butylhydroxyquinone, α-, β- or γ- tocopherols, tocopherol acetate, mixed tocopherols, ascorbic acid, β-carotene, rosemary extract, lycopene, propylgallate, erythorbic acid, ascorbyl palmitate etc.

For antibrowning agent the additive(s) is preferably selected from: ascorbic acid, SO_2 sources, such as sodium metabisulphite; or hexylresorcinol

For antimicrobials the additive(s) is preferably selected from: Benzoic, propionic and sorbic acids and their salts, DMDM Hydation, methyl, ethyl, propyl and butyl parabens, nisin, imidazolidinyl urea, methyl and methchloroisothiazolines, quaternary 15, diazolidinylurea, iodopropylbutylcarbamate, phenoxyethanol, bromonitrodioxane, 2-bromo-2-nitropropane-1, 3-diol. These additives are sold under various trade names such as Triclosan, Nipastat, Irgasan, Phenonip, Germaben, GermabenII, Bronopol, Kathion, Euxyl K100, Dowcil.

Preferably the additive is selected from one or more of citric acid, sorbic acid, ascorbyl palmitate and α-tocopherol.

The invention also provides a composition comprising a combination of two or more different compounds having the formula I.

In the compositions of the invention, the compound of formula I, or, where the compositions contain two or more compounds of formula I, at least one of the compounds, may be in encapsulated form.

Preferred embodiments of the invention will now be described with reference to the following illustrative examples.

Bioprocess for making compounds of formula I

In the following examples, analysis of compounds other than methoxyhydroquinone and protocatechuic acid was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	-	Spherisorb C-18
Mobile phase	-	60:40 deionised water: MeCN; 1% acetic acid
Flow rate	-	2 mlmin $^{-1}$
Detection	-	Ultraviolet at 290 nm.

Analysis of methoxyhydroquinone and protocatechuic acid was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	-	Spherisorb C-18
Mobile phase	-	80:20 deionised water: MeCN; 1% acetic acid
Flow rate	-	1.75 mlmin $^{-1}$
Detection	-	Ultraviolet at 290 nm.

In the following examples, where organisms are grown in culture broth, the growth medium can contain specified amounts of either, or both, a

vitamin supplement and a trace elements supplement. These were prepared as follows.

Vitamin supplement: biotin (2 mgl^{-1}), folic acid (2 mgl^{-1}), pyridoxine (10 mgl^{-1}), riboflavin (5 mgl^{-1}), thiamine (5 mgl^{-1}), nicotinic acid (5 mgl^{-1}), pantothenic acid (5 mgl^{-1}), vitamin B12 (0.1 mgl^{-1}), 4-aminobenzoic acid (5 mgl^{-1}), and thioacetic acid (5 mgl^{-1}).

Trace elements supplement: concentrated hydrochloric acid (51.3 mll^{-1}), MgO (10.75 gl^{-1}), $CaCO_3$ (2.0 gl^{-1}), $FeSO_4.7H_2O$ (4.5 gl^{-1}), $ZnSO_4.7H_2O$ (1.44 gl^{-1}), $MnSO_4.4H_2O$ (1.12 gl^{-1}), $CuSO_4.5H_2O$ (0.25 gl^{-1}), $CoSO_4.7H_2O$ (0.28 gl^{-1}), and H_3BO_3 (0.06 gl^{-1}).

Commercial supplies of *Sacharomyces cerevisiae* from Tesco plc, Sainsburys plc or Hovis yeast were used in Examples 2 and 8. *Candida versitalis* was obtained as NCYC 1433.

All other organisms were isolated using the soil isolation protocol described, unless indicated otherwise.

Patent Example	Identification of microorganism	Organism characteristics	Where isolated
1	*Rhodotorula glutinis* IMI 379894	Yeast with orange, mucoid colonies. Colony form: circular, entire margin convex elevation.	From air (Ashford, UK) onto a yeast malt agar plate.
3	See example 1	See example 1	
4	See example 1	See example 1	
5	*Paecilomyces variotti* IMI 379901	Filamentous fungus producing light brown powdery spores	From fruiting body of a fungus growing in Amazon in Brazil
6	*Aspergillus niger* IMI 379897	Filamentous fungus producing brown black spores	From mouldy coconut, Ashford, UK
13, 14	*Paenibacillus polymyxa* IMI 382464	Gram positive, group 2 bacillus with oval, centrally positioned endospore and a thick, ridged coat	Bird nesting site at Dorking, UK
Abbreviations IMI - International Mycological Institute, Egham, Surrey, UK NCYC - National collection of Yeast Cultures, Norwich, UK			

Soil Isolation Protocol

To 2ml deionised water was added approximately 100 mg soil. The resulting suspension was mixed thoroughly (vortex mixer) allowed to stand at room temperature (22°C for 1 hour followed by further mixing to distribute suspended material. The macroscopic solids were allowed to settle for approximately 10 minutes and the supernatant (100µl) was applied to a suitable medium (see below) in a 90mm petri dish using a spread plate technique. Plates were incubated at 28°C until colony development was observed.

For the isolation of fungi, soil supernants were spread plated onto a yeast malt medium comprising: 4g glucose, 4g yeast extracts, 10g malt extract per litre deionised water.

For the isolation of bacteria, soil supernatants were spread plated onto nutrient agar (Oxoid, Unipath Limited, UK)

Example 1: Preparation of Vinylguaiacol

A strain of *Rhodotorula glutinis* (IMI 379894) was cultured at 30°C by shaking at 200 rpm on a yeast malt medium containing (per litre of deionised water): glucose 4g; yeast extract 4g and malt extract 10g. After 40 hours incubation, ferulic acid was added to a final concentration of $2gl^{-1}$. The incubation was continued for a further 21 hours during which time the progress of the reaction was monitored by h.p.l.c. analysis using the conditions described above.

After 21 hours incubation the reaction had progressed to a molar conversion of 97.4%. The molar conversion after 3 hours was 61%.

Example 2: Preparation of Vinylguaiacol

Saccharomyces cerevisiae, "Bakers yeast", (2g, purchased from J. Sainsburys plc under the trade mark Sainsburys Easy Blend) was activated by suspending dry yeast powder in deionised water (20 ml) for 30 minutes at 37°C. A medium (1 l) containing (per litre deionised water): glucose 4g; yeast extract 4g and malt extract 10g was inoculated (5%) with the activated yeast suspension and incubated at 30°C with shaking at 200rpm

for 96 hours. After 96 hours incubation, the cells were harvested by centrifugation (15 minutes at 4000rpm), resuspended in 50ml of 0.9% (w/v) NaCl, and then disrupted by passage once through a cell disrupter (operating pressure 30,000psi). To 50ml of the resultant disrupted cell suspension was added ferulic acid at an initial concentration of $10gl^{-1}$. Also added at the same time was 50ml of Miglyol to form an upper organic layer to the biphasic biotransformation mixture. The progress of the reaction was monitored by analysis as described above.

After incubation at 30°C for 64 hours the reaction had progressed to a 92% conversion to vinylguaiacol.

Example 3: Preparation of Vinylguaiacol from Maize Fibre

Ferulic acid was released from maize fibre as follows. A 10g portion of maize fibre was shaken (200 rpm) at 30°C, overnight, in a conical flask with 100 ml of 1M sodium hydroxide solution. The resulting solution was acidified to pH 5.5 prior to the addition of 45 ml of a culture of *Rhodotorula glutinis* (IMI 379894) which had been grown on yeast malt medium in a 250 ml shake flask and incubated with shaking (200 rpm) at 30°C for 40 hours. At this time a concentration of 0.495 gl^{-1} ferulic acid was detected. The resulting suspension was itself incubated at 30°C with shaking (200 rpm) and the vinylguaiacol concentration monitored by hplc. After 10 minutes a 7.9% conversion of ferulic acid to vinylguaiacol was observed; after 1 hour there was a 29% conversion; after 20 hours a 93% conversion. The reaction mixture was extracted twice with 50ml of n-

hexane and the combined extracts dried and evaporated to yield 48mg of an oil comprising 84% vinylguaiacol.

Example 4: Preparation of Vinylguaiacol from Maize Fibre

Ferulic acid was released from maize fibre as follows. A 50g portion of maize fibre was shaken (200 rpm) at 30°C, for 15 hours, in a conical flask with 500 ml of 1M sodium hydroxide solution. The resulting solution containing 940 mg ferulic acid was neutralised by the addition of concentrated hydrochloric acid. This was added to 1 litre of a culture of *Rhodotorula glutinis* (IMI 379894) which had been grown on yeast malt medium in a 5 litre shake flask and incubated with shaking (200 rpm) at 30°C for 24 hours. The mixture was adjusted to pH 5.5 and 1 litre of n-hexane was added. The resulting two-phase system was mixed gently (80 rpm) at 30°C. After 24 hours the two liquid phases were separated and the aqueous re-extracted with 500 ml of n-hexane. The combined organic solvent phases, which contained 540 mg vinylguaiacol (75% yield) were dried and evaporated to yield an oil (740 mg) which was 65% vinylguaiacol by assay. This represents a 66% recovery of vinylguaiacol from ferulic acid.

Example 5: Preparation of Protocatechuic Acid from Caffeic Acid

To 400 ml of sterilised yeast malt medium (4g glucose; 4g yeast extract; 10g malt extract; made up to 1 litre with deionised water) was added glucose (40g) and caffeic acid (1g) and the resultant mixture was inoculated with spores of *Paecilomyces variotii* (IMI 379901) prior to

incubation at 30°C with shaking at 200rpm. Further aliquots of glucose (20g) were added at 24 hours, 72 hours and 96 hours. After 168 hours, hplc assay indicated that there were 630 mg total of protocatechuic acid present in the reaction system, representing a 74% molar conversion. The culture broth was extracted with ethyl acetate (900 ml) and assay showed that 527 mg of protocatechuic acid had been recovered along with 45 mg of unreacted caffeic acid. Evaporation of the dried solvent yielded 750 mg of a pale yellow gum which was resuspended in diethyl ether (100 ml) to give a red, granular, insoluble solid which was removed and the remaining solution evaporated to give 700 mg of recovered solid which was 67% protocatechuic acid by assay and 6% caffeic acid. This solid was dissolved in diethyl ether (10 ml) to which was then added a further 10ml of petroleum ether 40/60. Evaporation of this solution by blowing nitrogen over the solution gave a yellow oil from which the solution was decanted and evaporated to give a cream coloured solid (435 mg) which was 96.3% protocatechuic acid by assay.

Example 6: Preparation of Methoxyhydroquinone from Ferulic acid

Ferulic acid (2g) was added to a medium comprising: 50g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl; 0.2g $MgSO_4$; 0.15g $CaCl_2$; 1 ml trace element solution and 10 ml vitamins solution, made up to 1 litre with deionised water. The mixture was inoculated with a starter culture (50 ml) of *Aspergillus niger* (IMI 379897) "Zyl 768" which had been incubated for 24 hours in the same medium and the resulting mixture incubated at 30°C with shaking at 200 rpm in a conical flask. After 67 hours the Methoxyhydroquinone concentration was assayed by hplc as being approximately 1 gl^{-1}. The biomass was removed by filtration and the

solution was treated with aqueous Sodium hydroxide (2M) to adjust the pH to 6.8. The solution was then extracted with Ethyl acetate (1100 ml then 600 ml) and the combined organic phases dried and evaporated to dryness to yield Methoxyhydroquinone (1.09g; 76% molar conversion) as a dark brown oil.

Example 7: Preparation of vinylcatechol from caffeic acid

Bakers yeast, *Saccharomyces cerevisiae*, (3 g purchased from Tesco Stores Ltd under the trade designation Tesco Easy Blend Dried Yeast) was activated by suspending the dried yeast powder in 200 ml of a medium containing (per litre of deionised water): glucose 4 g; yeast extract 4 g and malt extract 10 g. The suspension was incubated at 30°C with shaking at 200 rpm for 24 hours at pH of ca 5.0. After 24 hours incubation, caffeic acid was added to an initial concentrations of 1 gl^{-1}. The progress of the reaction was monitored by analysis as described above. After incubation for a further period of 72 hours, an additional aliquot of caffeic acid was added at a concentration of 1 g L^{-1}.

Following incubation for a total of 144 hours after first adding caffeic acid, the reaction had progressed to a molar conversion of 95%.

Example 8: Preparation of vinylcatechol from caffeic acid with a whole cell preparation

Bakers' yeast (0.5g, purchased from Tesco Stores Ltd under the trade designation Tesco Easy Blend Dried Yeast) was activated by suspending the dried yeast powder in 50 ml of a medium containing (per litre of

distilled water): glucose 4g; yeast extract 4g and malt extract 10 g. The suspension was incubated at 30°C with shaking at 200 rpm for 50 hours without pH adjustment. After 50 hours incubation, caffeic acid was added at an initial concentration of $5gl^{-1}$. The progress of the reaction was monitored by analysis as described above. After incubation for a further period of 113 hours an additional aliquot of caffeic acid was added at a concentration of $5gl^{-1}$. Also added at the same time was 50 ml of miglyol to form an upper organic layer and thus from a biphasic biotransformation reaction mixture. After incubation for a further period of 48 hours, an additional aliquot of caffeic acid was added at a concentration of 10 gl^{-1}. Again after incubation for a further 48 hours, a final aliquot of caffeic acid was added at a concentration of $10gl^{-1}$.

Following incubation for a total of 233 hours after first adding caffeic acid, biotransformation of the batch-fed acid ($30gl^{-1}$) had progressed to a molar conversion of 95% based on the concentration of vinyl catechol detected in the miglyol layer. Subsequent extraction of the migylol layer with an equal volume of either polyethylene glycol (average molecular weight 2000) or 1,2-propane diol (propylene glycol) resulted respectively in 88% and 76% partitioning of the vinylcatechol out of miglyol into the alternative organic solvent.

Example 9: Preparation of vinylcatechol from caffeic acid with a disrupted cell preparation

Bakers' yeast (0.5g, purchased from Tesco Stores Ltd under the trade designation Tesco Easy Blend Dried Yeast) was activated by suspending the dried yeat powder in 100ml of a medium containing (per litre of

distilled water): glucose 4g; yeast extract 4g and malt extract 10g. The suspension was incubated at 30°C with shaking at 200 rpm for 50 hours without pH adjustment. After 50 hours incubation, the cells were harvested by centrifugation (15 minutes at 4000 rpm), resuspended in 20ml phosphate buffer (0.1M, pH 5.85), and then ruptured by passage once through a cell disrupter (operating pressure 30,000 psi). The resultant disrupted cell suspension was made up to a total volume of 100ml with additional phosphate buffer (0.1M, pH 5.85). Caffeic acid was added at an initial concentration of $30gl^{-1}$. Also added at the same time was 100ml of Miglyol to form an upper organic layer and thus a biphasic biotransformation reaction mixture. The progress of the reaction was monitored by analysis as described above. After incubation for a period of 22.5 hours, an additional aliquot of caffeic acid was added at a concentration of $30gl^{-1}$.

Following incubation for a total of 94 hours after first adding caffeic acid, biotransformation of the batch-fed acid ($60gl^{-1}$) had progressed to a molar conversion of 94% based on the concentration of vinyl catechol detected in the miglyol layer.

Example 10: Preparation of vinylcatechol from caffeic acid with immobilised biocatalyst in a monophasic organic solvent

Bakers' yeast (3g, purchased from Tesco Stores Ltd under the trade name of Tesco Easy Blend Dried Yeast) was added to 2.5 litres of medium containing (per litre of distilled water): glucose 4g; yeast extract 4g and malt extract 10g. The suspension was incubated at 30°C with shaking at 200 rpm for 120 hours without pH adjustment. After 120 hours

incubation, the cells were harvested by centrifugation (15 minutes at 4000 rpm), resuspended in 50 ml of 0.9% (w/v) NaCl, then disrupted by passage once through a cell disrupter (operating pressure 30,000 psi). To 25 ml of the resultant disrupted cell suspension was added 25 ml of a sterile solution of 3.0% (w/v) sodium alginate. After through stiring, the mixture was added drop-wise to 200 ml of 0.2M $CaCl_2$, and the resultant beads allowed to harden for 18 hours at 4°C. The hardened beads were washed with 0.9% (w/v) NaCl and added to 25 ml of miglyol containing 1.0g caffeic acid (ie 40g/L). The suspension was incubated at 30°C with shaking at 200 rpm, and progress of the biotransformation monitored as described above.

After incubation at 30°C for 70 hours the reaction had progressed to 74% molar conversion based on the concentration of vinylcatechol detected in the miglyol.

Example 11: Production of Ethylguaiacol from ferulic acid using C. versitalis

Candida versitalis (NCYC 1433) was grown from a plate culture inoculum for 6 days in yeast malt medium containing 10g/L malt extract, 4g/L yeast extract, 4g/L glucose, and 2% sodium chloride dissolved in deionised water and autoclaved at 120°C. The 50ml culture was incubated at 30°C and 200 rpm in a 250ml conical flask.

After 6 days this culture was used to provide a 10% inoculum for a 150ml second culture of the yeast malt medium occupying 50% v/v of the flask. This was incubated at 21-22°C for 24 hrs while agitating at 150 rpm.

Then ferulic acid was added to a concentration of 2g/L, together with 100 ml of Miglyol, which alternatively could be added after 50 hrs when the concentration of ethylguaiacol in the aqueous phase had reached 0.25-0.3g/L. (Miglyol is added because the strain appears to be intolerant of the ethylguaiacol product, with the maximum concentration of ethylguaiacol accumulated (in a monophasic reaction) in the absence of Miglyol as product sink being 0.5 g/L).

Ethylguaiacol formation was monitored by hplc using as solvent 60:40 water:acetonitrile plus, 1% acetic acid, at a flow rate of 2ml/min and monitoring at 290 nm. Ethylguaiacol was formed in a good yield from ferulic acid, with vinylguaiacol being detected as the intermediate. After 184 hrs incubation, the concentration of ethylguaiacol in the Miglyol was 3.64g/L, which represents 92 to 94% of the theoretical maximum yield. The ethylguaiacol could be easily recovered from the Miglyol as a pure chemical by solvent extraction into hexane and then rotary evaporation to dryness.

Example 12: Extraction of Protocatechuic acid from onion skins

Chopped onion waste material (200g dry weight) was suspended in 0.1M NaOH (1l). This 20% w/v suspension was heated at 90°C for 4 hours in a water bath. The suspension was then pressed to remove the solid material and the solids were washed with sufficient deionised water to return the volume of the liquor to 1l. The liquor contained 0.7g/l protocatechuic acid (PCA); therefore the yield as a percentage of the dry material used was 0.35% w/w. The liquor was re-heated to 90°C. Sodium hydroxide (10M) was added to a final concentration of 0.1M, then

chopped waste onion material (200g) was suspended in the liquor, and this suspension was again heated at 90°C for 4 hours. The suspension was pressed and the solids were washed as before. The 1l of liquor contained 1.17g/l PCA. The overall release efficiency in terms of dry weight yield was therefore 0.29%. This reloading was repeated two more times to achieve a final PCA concentration of 2.76g/l in the liquor corresponding to an overall yield of 0.32% w/w of dry onion material added.

Extraction of PCA from onion skin liquor

Reloaded onion skin liquor containing 2.3g/l PCA (1l total, 2.30g PCA), was adjusted to pH 3 with concentrated HCl and centrifuged at 4000rpm for 20 minutes. The resultant supernatant totalled 910ml and contained 2.35g/l PCA (2.14g PCA). The clarified aqueous layer was extracted with an equal volume of n-butyl acetate. After 24 hours the aqueous layer contained 0.48g/l PCA (20% of the original concentration). Therefore the organic layer contained 1.84g/l PCA (910ml total, 1.68g PCA). The solvent was then removed *in vacuo* to leave a solid (2.75g) which was shown to be 60% PCA (by HPLC).

***Example 13: Preparation of vinylguaiacol from ferulic acid using* Paenibacillus polymyxa**

Paenibacillus polymyxa (Zyl 277; IMI CC Deposit No 382464) cells were grown at 30°C, shaking at 200 rpm for 27 hours on a medium comprising per litre deionised water: $(NH_4)_2$ SO_4, 5g; K_2HPO_4, 2g; NaCl, 0.2g; glucose, 10g; malt extract, 3g; yeast extract, 3g; $MgSO_4$, 0.22g; $CaCl_2$, 0.015g; ferulic acid, 0.5g. The cells were harvested by centrifugation (4,000 x g 15 m') washed with 0.9% (w/v) saline solution followed by

resuspension in 0.9%(w/v) saline solution as a 20-fold concentration. An aliquot of concentrated cells (5ml) was added to a solution of sodium alginate (15ml 3.5% w/v) and mixed thoroughly, prior to addition dropwise from a 3ml plastic pipette into 1 litre of 0.2M $CaCl_2$ solution. The beads formed by this procedure were stored at 4°C overnight in $CaCl_2$ solution to harden before washed in 2l of tap water.

The beads interspersed with an inert packing material were packed into a 100ml glass column. A solution of ferulic acid in tap water (500 ml, 6g/l) was pumped continuously through the column at a temperature of 24°C and the pH of this solution was maintained at pH 7.0. After 6 hours operation, the aqueous stream exiting the top of the column was continuously extracted into hexane (500 ml) to remove vinylguaiacol, prior to returning to the column.

Results

Vinylguaiacol concentrations (gl^{-1}) were:

Time (Hr)	Aqueous	Hexane	Total
1.75	0.074	-	0.074
3	0.116	-	0.116
6	0.175	-	0.175
23	0.101	0.8	0.901
48	0.144	1.332	1.476
122	0.152	2.09	2.24

Example 14: Production of Vinylguaiacol from Ferulic Acid by **Paenibacillus polymyxa** *(ZYL277) in a Two Phase System*

Paenibacillus polymyxa (Zyl 277; IMI CC Deposit No 382464) was grown in a bioreactor in a medium containing (g/l) $(NH_4)_2SO_4$, 5; K_2HPO_4, 2; NaCl, 0.2; yeast extract, 2; malt extract, 2; glucose, 10; ferulic acid, 0.5; 10ml/l of a solution containing 0.1M $MgSO_4$/0.01M $CaCl_2$; at 30°C, pH 6.0, oxygen 70% on a stirrer cascade (100-500 rpm).

After 24h, 100 ml of culture was placed in a 250ml conical flask, stirred at 25°C with pH control at 7 using 2M NaOH or dilute phosphoric acid as required. 4 g/l ferulic acid (free acid) was added to the aqueous phase before it was overlaid with 100ml hexane. The hexane was added to partition vinylguaiacol from the aqueous phase where it may be toxic to the organism. Vinylguaiacol concentrations in both the aqueous and hexane phase were determined by HPLC. Further ferulic acid was added to the aqueous phase as the reaction proceeded. The hexane layer was removed periodically and replaced with 100ml of new hexane to prevent it becoming saturated with vinylguaiacol. Vinylguaiacol concentrations in both phases at the time of changing the hexane phase are shown below along with the cumulative total ferulic acid added to the aqueous phase (g/l).

Time (h)	Vinylguaiacol (g/l)			Total Ferulic Acid
	Aqueous	Hexane	Total	Added (g/l)
21	0.70	9.78	10.48	12
93	0.78	9.86	10.64	22
117	0.70	10.44	11.14	34
143	0.60	7.94	8.54	42
172	0.37	7.30	7.67	50
262	0.62	10.58	11.20	58

314	0.53	9.40	9.93	66

The seven collected hexane layers contained a total of 5.42g vinylguaiacol. Ferulic acid additions had been carried out to take account of increases in aqueous volume due to pH control. In total 7.16g ferulic acid had been added (equivalent to 66 g/l taking account of increasing aqueous volume). This equates to a molar yield for vinylguaiacol of 98%.

Table 1: Preparation of compounds of the invention from caffeic acid or ferulic acid and selected microorganisms.

Product	Reaction molar yield % (g substrate supplied l^{-1})	Product g l^{-1} culture	Recovered molar yield (%)	Productivity of biocatalyst (g product g $cells^{-1}$ dry wt.)	Approx. yield from 500l fermenter (kg)	Zyl No.
Ferulic acid to vinylguaiacol	94 (8.5)	6.1	87	0.95	2.4	702
Caffeic acid to vinylcatechol	90 (1.5)	#1.0	68	n.d	0.4	739
Caffeic acid to Protocatechuic acid	51 (4)	1.75	n.d	n.d	0.88	733
Ferulic acid to ethylguaiacol	92-94	3.64	n.d	n.d	0.73	NCYC 1433
Ferulic acid to methoxyhydro-quinone	83 (5)	#3.0	~70	n.d	1.5	733

-Indicates a minimum value where further addition of substrate has not been investigated.

Antimicrobial activity

Example 15: Antimicrobial assay method

The minimum inhibitory concentration (MIC) of test compounds was determined at 30°C in microtitre plates (24 well) using a static culture technique.

The following organisms were used as assay test strains:

S. aureus NCIMB 9518
E. coli NCIMB 8879
B. cereus NCIMB 11843
P. aeruginosa NCIMB 8626
C. albicans DSM 5817
A. niger IFO4414
A. flavus
S. mutans ATCC 25175
Listeria monocytogenes DSM 20600
P. acnes ATCC 6919.
S. choleraesuis
P. ovale

All bacteria were cultured on nutrient broth per litre of deionised water: (Oxoid, pH 7.4) and all fungi on a yeast medium comprising per litre of deionised water: malt extract 10g; yeast extract 4g; and glucose 4 g. Ethanol was used as a solubilising agent to prepare stock solutions (0.1 gml^{-1}) of each test compound. The final concentration of ethanol in

all growth studies was corrected to a maximum of 2%. Microtitre wells containing growth medium (2 ml) and an appropriate concentration of test compound were inoculated with either 5µl of a 15 hour seed culture (bacteria and yeast strains) or with a 1µl loopful of fungal spores. A visual assessment of growth was carried out at 24 hr incubation for bacteria and yeast strains and at 48 hr for filamentous fungi. The MIC was recorded as the concentration (mgl^{-1}) at which no growth of the organism was observed or the concentration at which fungal spore germination was completely inhibited.

The results are shown in Table 2.

Table 2 - Antimicrobial activity of vinylguaiacol and vinylcatechol

	Vinyl-catechol	Vinylcatechol plant extract	Vinyl-guaiacol	Vinylguaiacol plant extract (65% VG)	Vinyphenol	Ethyl-guaiacol	Methoxy-hydroquinone	Methyl-parabens	Propyl-parabens
Gram Positive Bacterial									
Bacillus cerus	600	-	800	300	800-1,000	1,200	500	>1,500	400
Streptococcus mutans	400	600	600-1,000	400	-	1,000	600-1,000	800	400
staphylococcus aureas	600	-	800	>1,200	200-400	1,600	-	>2,000	600
Listeria monocytogenes	600-1,000	600-800	800-1,000	1,000	800	>1,000	-	>1,000	400
Gram Negative Bacteria									
Pseudomonas aeruginosa	600-800	-	2,000	1,700	800	1,600	>1,000	2,000	Inactive (insoluble)
Propionibacterium acnes	400-1,000	600-800	600-1,000	1,000	500-800	>1,000	>1,000	>1,000	400
Escherishia coli	600	-	500	900	-	800	-	1,000	500
Salmonella choleraesuis	400	400	600	600	600	-	-	>1,000	-
Yeasts									
Candida albicans	450	-	500	900	-	500	>1,000	800	200
Pityosporium ovale	<1,000	800	400	400-1,000	-	-	-	>1,000	-
Fungi									
Aspergillus flavus	450-600	-	200	300	>1,000	500	>1,000	1,000	400
Aspergillus niger	500	-	200	300	-	500	>1,000	500	400

Biotest 1

Two agar discs overgrown with mycelium of a test fungi were placed 10 mm from the centre of 30 mm petri dishes which had been coated with 2.5 ml of the appropriate agar media. Following this, 50 µl of a pure liquid test compound or a saturated solution of a test compound in sterile water was placed in a prepared 9mm-hole in the centre of each dish. The dish was then dried in a laminar box. A control dish was treated with sterile water instead of the test compound. After an incubation period of 3, 5 and 7 days at 22°C the radial growth of fungi colonies and the inhibition zones arising from the centre of petri dishes that had been treated with a test compound were measured by comparison with the nontreated control. The bordering mycelium in the direction of the inhibition zone was examined for irregular growth by light microscopy.

The variable reaction of the tested fungi to the test compounds is shown in Table 3, below. Of the test compounds EG, VC and VG exhibited strong inhibitory effects against all fungal species tested. Other test compounds such as MHQ or PCA had a selected inhibitory effect against one or two fungal species. The relatively poor inhibitory effect of CA is attributed to its poor solubility in the different media. The growth of *Alternaria brassicae, Botrytis cinerea, Fusarium culmorum and Alternaria dauei* was highly susceptible to and inhibited by most agents. *Septoria tritici* appeared to be more tolerant against the test compounds.

Table 3: **The inhibition zones (mm) of various fungi species after 9 days incubation with 0.025 ml of the various test compounds in 2.5 ml agar (average of two repetitions).**

Test Organism	Control	CA	EG	MHQ	PCA	VC	VG	VP
Alternaria brassicae	0	3,8	19,3	11,7	9,8	21,8	22,7	10,7
Alternaria dauci	0	1,5	22,2	8,8	0,3	10,3	21,2	10,3
Botrytis cinerea	0	0	22,3	19	1,2	25,3	22,0	10,7
Drechslera tares	0	0,2	20,2	8,5	0	13,1	9,7	0
Fusarium culmorum	0	0,4	20,1	19,5	16,8	18,6	9,3	16,7
Septoria nodbrum	0	0,4	19,8	9,5	0	15,8	12,1	2,1
Septoria tritici	0	0	8,1	8,3	0	10,4	10,8	0

Key: CA - Caffeic acid
PCA - Protocatechuic Acid
VP - Vinylphenol
EG - Ethylguaiacol
VC - Vinylcatechol
MHQ - Methoxyhydroquinone
VG - Vinylguaiacol

Biotest 2

Agar media was autoclaved and after cooling down to 40°C it was mixed with amounts of a pure liquid test compound or saturated solution of a test compound in sterile water so that the resultant agar media had a concentration of 2000, 1000, 500, 100, 50 or 10 ppm of pure test compound molecules in the agar media. After cooling and drying the agar media, 100 µl of fungal spore suspensions were applied and spread over the agar surface. After an incubation of 1,2,3,5,7, and 9 days at 22°C the germination of fungal spores were observed by light microscopy. The amount of germinating spores in defined square sectors were counted and

the maximum inhibitory concentration of the test agents was assessed by comparing the proportion of germinating spores to a 50% inhibition of spore germination at a specific concentration of the test compound. This ratio was defined as MID.

The intensity of spore germination after 9 days incubation after dissolving adequate amounts of various test compounds in 5 ml agar (average of two repetitions) is shown in Table 4. ++ indicates that more than 50% of spores germinated, + indicates that less than 50% of spores germinated, and - indicates that no or only single spores germinated.

Table 4

Test Organism	Conc. (ppm)	Contr	CA	EG	MHQ	PCA	VC	VG	VP
Alternaria brassicae	2000	++	+	-	-	-	-	-	-
	1000	++	++	-	+	+	-	-	+
	500	++	++	-	++	++	-	-	++
	100	++	++	-	++	++	+	++	++
	10	++	++	++	++	++	++	++	++
Alternaria dauci	2000	++	++	-	-	-	-	-	-
	1000	++	++	-	+	+	-	-	-
	500	++	++	-	++	++	-	-	+
	100	++	++	-	++	++	-	+	++
	10	++	++	+	++	++	++	++	++
Botrytis cinerea	2000	++	++	-	-	-	-	-	-
	1000	++	++	-	+	-	-	-	-
	500	++	++	-	++	++	-	+	++
	100	++	++	-	++	++	+	++	++
	10	++	++	++	++	++	++	++	++
Drechslera teres	2000	++	++	-	-	++	-	-	++
	1000	++	++	-	+	++	+	-	++
	500	++	++	-	++	++	++	-	++
	100	++	++	-	++	++	+	+	++
	10	++	++	++	++	++	++	++	++

Fusarium culmorum	2000	++	-	-	-	-	-	-	+
	1000	++	++	-	+	+	-	-	++
	500	++	++	-	++	++	-	-	++
	100	++	++	-	++	++	+	+	++
	10	++	++	++	++	++	++	++	++
Septoria nodoram	2000	++	++	-	-	++	-	-	++
	1000	++	++	-	+	++	-	+	++
	500	++	++	-	++	++	-	++	++
	100	++	++	-	++	++	+	++	++
	10	++	++	++	++	++	++	++	++
Septoria tritici	2000	++	++	-	-	++	-	-	++
	1000	++	++	-	+	++	+	+	++
	500	++	++	-	++	++	++	++	++
	100	++	++	-	++	++	++	++	++
	10	++	++	++	++	++	++	++	++

Key: CA - Caffeic acid
PCA - Protocatechuic Acid
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MHQ - Methoxyhydroquinone
VG - Vinylguaiacol

Microscopic investigations of fungal spores after application of the test compound containing agar media showed relative homogeneous reactions concerning germination in both duplicate tests. EG exhibited a strong inhibitory effect against all spores of tested fungi. MHQ and VG induced irregular growth of germinating *Botrytis*-mycelium with increased numbers of vacuoles. VC degraded the spores of *Fusarium* very strongly. Spores of *Alternaria* spp., *Botryris, Fusarium* were highly degraded, with swollen vacuoles.

Biotest 3

Agar media was autoclaved and after cooling down to 40°C it was mixed with amounts of a pure liquid test compound or a saturated solution of a test compound was added so that the resultant agar media had a concentration of 2000, 1000, 500, 100, 50 or 10 ppm of pure test compound molecules in the agar media.

After this mycelium of a test fungi was placed in the centre of petri dishes. After an incubation period of 3, 7 and 11 days the radial growth of the fungal colonies that had been treated with a test compound were measured by comparison with a nontreated control. The mycelium were examined by light microscopy.

The radial growth (mm) of a mycelium of test fungi after 9 days incubation with various test compounds in 5 ml agar (average of two repetitions) is shown in Table 5. In Table 5, the sign - represents no fungal growth.

TABLE 5

Test Organism	Conc. (ppm)	Contr	CA	EG	MHQ	PCA	VC	VG	VP
Alternaria brassicae	2000	55	51	-	18	28	-	-	22
	1000		56	-	42	39	-	-	36
	500		55	23	55	52	12	16	57
	100		54	36	56	54	25	49	54
	10		55	54	54	54	56	55	56
Alternaria dauci	2000	62	64	-	25	28	-	-	-
	1000		62	-	38	46	-	-	31
	500		63	-	64	63	-	22	47
	100		65	11	63	62	14	48	63
	10		63	43	61	62	60	62	61

Botrytis cinerea	2000	77	79	-	35	-	-	-	-
	1000		81	-	68	24	-	11	27
	500		76	-	77	78	32	44	78
	100		78	24	80	77	77	76	76
	10		77	75	78	77	77	76	78
Drechslera teres	2000	36	36	-	12	35	25	-	35
	1000		34	-	36	35	30	-	37
	500		36	-	37	35	37	14	38
	100		35	26	27	36	37	28	36
	10		35	37	35	36	36	35	37
Fusarium culmorum	2000	85	72	-	45	56	-	-	71
	1000		82	-	68	75	-	-	84
	500		85	-	83	87	22	32	84
	100		85	33	85	87	46	65	87
	10		86	82	84	88	85	88	85
Septoria nodoram	2000	28	29	-	12	28	-	22	28
	1000		27	-	23	28	-	26	29
	500		28	-	27	30	17	28	26
	100		27	15	28	28	23	28	28
	10		26	27	28	29	27	27	27
Septoria tritici	2000	44	42	-	21	45	17	24	46
	1000		42	-	36	44	26	32	45
	500		44	-	43	44	44	45	44
	100		45	33	42	46	43	44	46
	10		45	44	44	42	45	45	44

Key: CA - Caffeic acid
PCA - Protocatechuic Acid
VP - Vinylphenol
EG - Ethylguaiacol
VC - Vinylcatechol
MHQ - Methoxyhydroquinone
VG - Vinylguaiacol

Biotest 4 - Screening of phytotoxic effects after application on leaf material

Surface sterilised leafs of tomato, cabbage or potato were placed on wet filter paper in petri dishes. The test compounds were diluted in acetone water or ethanol water (1ml, 70%v/v.20°C) to get concentrations of 1.000 or 2.000 ppm. The solutions were diluted by ten-fold dilution steps using

sterile distilled water with 0,02% Tween 20 (used to reduce the surface tension of water). 5 mm filter paper discs were soaked with 25 µl of the diluted solution of test compound or with a saturated solution of the test compound in sterile water. The soaked filter paper discs were placed on the leaf surfaces and the petri dishes were shut to prevent evaporation. After 12, 24 and 48 hours the area of the leaf under and next to the paper discs was examined and any lesions, chloroses or other irregularities were recorded.

Biotest 5 - Screening of phytotoxic effects after application on leaf material

The test compounds were diluted acetone water or ethanol water (1ml, 70%v/v.20°C) to get concentrations of 1.000 or 2.000 ppm. The solutions were diluted by ten-fold dilution steps using sterile distilled water with 0,02% Tween 20 (used to reduce the surface tension of water). 5 mm filter paper discs were soaked with 25 μl of the diluted solution of the test compound or with a saturated solution of the test compound in sterile water. The soaked paper discs were placed on the surfaces of the leafs of living tomato, cabbage or potato plants in a greenhouse. The leafs were wetted with a water sprayer 3 times a day, to prevent the filter paper discs from drying out. After 12, 24 and 48 hours the area of the leaf under and next to the paper discs was examined and any lesions, chloroses or other irregularities were recorded.

The phototoxical effects of test agents against three test plants (tomato, cabbage and potato) after application of the test compounds onto leaf surface are shown in Table 6 below. These results represents an average

of the results from Biotest 4 and 5. The sign ++ indicates a strong chlorotic or necrotic effects, + indicates less chlorotic or necrotic effects, and - indicates no effects.

TABLE 6

Test Plant	Dilution	CA	EG	MHQ	PCA	VC	VG	VP
Cabbage	1/0	-	++	++	-	+	-	++
Potato		-	++	++	-	++	-	++
Tomato		-	++	++	-	++	-	++
Cabbage	1/10	-	+	+	-	+	-	+
Potato		-	+	+	-	+	-	+
Tomato		-	+	+	-	+	-	+
Cabbage	1/100	-	+	-	-	-	-	-
Potato		-	+	-	-	-	-	-
Tomato		-	+	-	-	-	-	+
Cabbage	1/1000	-	-	-	-	-	-	-
Potato		-	-	-	-	-	-	-
Tomato		-	-	-	-	-	-	-

Key: CA - Caffeic acid
PCA - Protocatechuic Acid
VP - Vinylphenol
EG - Ethylguaiacol
VC - Vinylcatechol
MHQ - Methoxyhydroquinone
VG - Vinylguaiacol

The two simple biotests were able to give a general impression about possible phytotoxic side effects of the test compounds. More detailed investigations of the damaged leaf areas showed that:

MHQ initiated lytic processes below the cuticula;

VC destroyed cuticula; and

VG damaged cuticula and initiated cell lysis

These lesions developed under the filter paper discs only; the neighbouring leaf area had not been effected. Caffeic acid developed salty deposit's on the leaf surface. The degree of damage was proportioned to the concentration of the agent used.

The above results demonstrate that the compounds of formula VIII, formed by the biotransformation processes, exhibit strong antifungal activities against various fungal species, with MID/MIC in the range of 10-100 mgl^{-1}. Such an MID is significantly below the concentration of the test compound required to cause substantial phytotoxic effects on the test range of plant materials. The fungicides identified by the present invention are small non-protein molecules which are likely to be stable in field-use conditions.

Antioxidant activity

Example 16: Free radical antioxidant assay method

This method, based on the use of diphenylpicrylhydrazyl hydrate (DPPH), is adapted from the method of Brand-Williams, Cuvelier & Berset, *Lebesm-Wiss.u.Technol.*, 1995, **28**, 25. A cuvette containing 1.4 ml of a 60 μM solution of DPPH in methanol was treated with a solution of test compound in methanol at various concentrations and the decrease in UV absorbance, measured at 515 nm, was monitored until the spectrum reached a plateau. This allowed calculation of the concentration of the test compound to quench all or half of the free radical species present.

Example 17: Peroxidation inhibition assay method for antioxidant activity

This method is based on the inhibition of the oxidation of fats or oils and is adapted from the method of Marinova & Yanishlieva, *Fat Sci. Technol.*, 1992, **94**, 428. Peroxide values were determined by the method described in the AOCS Official Method Cd-8-53, 1960 reapproved, 1973. Test antioxidant compounds (0.1 mgg^{-1}) were added to lard (100g) in an Erlenmeyer flask (250 ml) and the flasks incubated at 60°C with shaking at 260 rpm. The peroxide value was determined over a timecourse and compared with a control flask containing no test compound. Time to "failure" was measured and this was regarded as the time when the lard reached a peroxide value of 140 milliequivalents per 1000g. Assays using soyabean oil as the target for oxidation were carried out at both 60°C or at 130°C with vigorous stirring and time to "failure" was taken as 100 milliequivalents per 1000g.

Antioxidant Activities

Table 7

Material/Molecule	Fat/Oil rancidity (days to failure)	Free-radical scavenging activity (mg/L)	Rate of free radical scavenging
Vinylcatechol from sunflower meal	77	0.34 (1 equal)	87.9
Vinylcatechol	39	0.7 (5)	35.1
Methoxyhydroquinone	17-28	1.05-1.3 (6)	38.9
Protocatechuic acid	9	0.6 (4)	6.2
Vinylguaiacol	3	3.5 (13)	7.8
Ethylguaiacol	3	1.98 (10)	3.8
Standards			
Propylgallate +	28	1.06	28.3
Butyrated hydroxytoluene +	25	2.6	0.3
Tertiary butylhydroquinone +	23	1.5	5.7
Butyrated hydroxyanisole +	18	1.6	1.23
α-tocopherol +	9	5.4	3.8

Bracketed numbers indicate the ranking of the experimental free-radical scavenging activities. This is the concentration of active material necessary to achieve 50% inhibition of activity. The rate of free radical scavenging is measured in terms of the number of moles of DPPH consumed per minute per milligram of antioxidant.

Antibrowning activity

Example 18: In-Vitro Antibrowning assay

To 480µl of a solution of chlorogenic acid (100 $\mu g ml^{-1}$) in phosphate buffer (100 mM, pH 6.2) was added a solution of the test compound (100 $\mu g ml^{-1}$) in the same buffer in a cuvette, and the mixture kept at 30°C. The mixture was made up to 1170 µl with phosphate buffer and a reaction initiated by the addition of tyrosinase (30µl, 1000 units ml^{-1}). The reaction was monitored by monitoring the increase in absorbance in the UV spectrum at 265 mm over 3 minutes. The amount of compound in the test solution was varied over a range such that a concentration giving 50% inhibition of tyrosinase activity could be determined. A similar assay to measure inhibition of laccase comprised adding a syringaldazine solution (0.15 ml, 0.216 mM solution in methanol) to phosphate buffer at 30°C and the reaction monitored at 530nm in the UV.

Example 19: Antibrowning assay

The ability of vinylguaiacol to prevent the browning of lettuce, apple, potato, banana and avocado was assessed. Small pieces of each vegetable (approximately 5mm square) were immersed for 5 minutes in an aqueous solution of each compound (1ml of 1.0 mg ml^{-1} or 0.1 mg ml^{-1}) and in combination with known antibrowning agents, such as sodium metabisulphite, ascorbic acid, and 4-hexylrescorcinol. The inhibition of browning was assessed periodically over 24 hours by visual comparison with pieces of lettuce, apple and avocado that had been either untreated or immersed in water for 5 minutes. The browning of pieces of fruit were assessed using a percentage scale with the control pieces of fruit having a value of 100%. These assays demonstrated that vinylguaiacol prevented browning of lettuce, apple, potato, banana and avocado.

Skin Lightener Assay Method:

Tyrosine: To 171.4µl of a solution of tyrosine (1.5mM) in phosphate buffer (100mM, pH6.4) was added of an inhibitor solution (100µg ml^{-1}) in phsophate buffer (100mM, pH6.4) in a quartz cuvette. The reaction mixture was made up to 980µl with phosphate buffer (100mM, pH6.4) and the reaction initiated by the addition of tyrosinase (Sigma, 220 µl, 1100 units/ml in phosphate buffer (100mM, pH6.4)), The reaction was monitored by the increase in absorbance at 470nm over 10 minutes.

Laccase Assay Method:

To 2.20 ml of phosphate buffer was added 0.5ml of laccasse enzyme solution (25-50 units/ml in water) and 0.3ml of syringaldazine solution (0.2166mM in ethanol). The reaction was monitored by the increase in absorbance at 530nm over 10 minutes.

Table 8

In Vitro Antibrowning Data

Tyrosinase Inhibitor	Degree of inhibition IC_{50}* (μg/ml)	Laccase Inhibition
Vinylguaiacol Plant extract 65%	2.4 4.4	Yes
Salicylhydroxamic acid +	0.24	
Kojic acid +	2.3	
Ascorbic acid +	4.1	
Sodium metabisulphite	4.3	

*Concentration of inhibitor to inhibit tyrosinase activity by 50%

Table 9

In vitro Skin Lightening Data

Tyrosinase Inhibitor	**Degree of Inhibition IC_{50}* (μg/ml)**
Vinylguaiacol	21.5
Ethylguaiacol	40
Hydroquinone +	2.27
Bearberry extract +	3.1
Bearberry extract +	4.4
Kojic acid +	6.5

*Concentration of inhibitor required reducing tyrosinase activity to 50%

+Standards

Table 10

Solubilities, taste, odour and colour of compounds of the invention

Molecule	Solubility (g/l)					Taste/ Odour[1]	Colour (Solution in Ethanol[1])
	Water (pH 7.0)	Ethanol	Oil	Ethylene Glycol	Propylene Glycol		
Vinylcatechol	>2	>100	1	500	---	None	Colourless
Vinylguaiacol	>2	>100	>50	---	---	Cloves	Colourless
Protocatechuic acid	>300	>500	<0.2	250	---	None	Colourless
Methoxyhydroquinone	>300	>1000	<0.2	333	---	None	Very pale brown
Ethyguaiacol	ND	ND	ND	250	---	Smoky	Colourless

[1] Determined at effective concentrations

ND Not Determined

Compositions of the invention

For the purposes of this invention "cosmetic products" are products intended for increasing the appeal, visually and olfactively, of the human body. Likewise "personal care products" are products intended for cleaning, smoothing or otherwise improving the health and well-being of the outside of the human body. These definitions of cosmetic and personal care products at least partially overlap since many products provide functions in both categories. Examples of such products are: perfumes and like products known as "eau de toilette" and "eau de parfum", hand and body lotions, skin tonics, shaving products, bath and shower products, deodorant and antiperspirant products, hair care products such as shampoos and hair conditioners, mouth and dental care products. Such products are well known in the art. Thus, examples of skin care products are described in "Harry's Cosmeticology", R. G. Harry, 6th edition, Leonard Hill Books (1973), Chapters 5-13, 18 and 35; examples of deodorants and antiperspirants are described in C. Fox, cosmetics and Toiletries **100** (Dec. 1985), pp 27-41; examples of hair care products are described "Harry's Cosmeticololgy", vide supra, chapters 25-27; examples of dental care products are described in M. Pader, Oral Hygiene: Products and Practice, Marcel Dekker, New York (1988). Cosmetic and personal care products are usually perfumed, on the one hand to give pleasant odour to the products themselves and on the other hand to have the body parts to which they are applied emit a pleasant odour after their use.

Moreover, the compounds of the invention may be used in food and beverage compositions in addition to or instead of conventional preservatives.

Tables 11 to 14: Personal Care Products including the compounds of the invention

Table 11 : A photostable sunscreen lotion

Ingredient	% w/w
Glyceryl monomyristate	5.00
Cetyl alcohol	2.0
Butylmethoxy dibenzoylmethane	1.00
Isopropyl myristate	7.00
Oleoyl alcohol	3.00
Polysiloxane A	5.00
DEA cetylphosphate	3.00
Water, deionised	67.00
Propylene glycol	6.00
A compound of the invention	0.60
Fragrance	0.30

Table 12

Shampoo with behenic acid + silicone

Ingredient	% w/w
PVP	0.50
Ammonium laureth sulfate; 30% active	10.00
Ammonium lauryl sulfate; 30% active	50.00
Distearyldimonium chloride (Varisoft TA 100)	0.30
Cocamide DEA	1.50
Montah Wax (contains 5% behenic acid)	1.25
Lauryl betaine; 40% active	2.00
Dimethiconol/dimethicone (Blend 33% gum / 67% oil)	2.50
Fragrance	0.50
A compound of the invention	0.08
Water, deionised	to 100%

Table 13

All purpose dry skin cream

Ingredient	%w/w
Phase A (80°C)	
Glyceryl stearate SE	12.00
Stearyl stearate (Hetester 412)	4.00
Di-C 12-15 Alkyl fumarate (Marrix SF)	2.00
Ganex V220	2.00
Minno 21	6.00
Phase B (80°)	
Water, deionised	63.00
Glycerin	10.00
Phase C	
A compound of the invention	1.00

Add Phase A to Phase B. Mix at 80°C. Cool to 60°C and add Phase C. Cool to 50°C and package.

Table 14

Moisturiser with sun protection

Ingredient	%w/w
Phase A (45°C)	
Water deionised	63.40
Phase B (dry blend)	
Magnesium aluminium silicate (Veegum Reg.)	1.00
Xanthan Gum	0.50
Phase C (45°C and dissolve)	
Propylene glycol isoceteth-3 acetate (Hetester PHA)	10.00
Octyl methoxycinnamate	7.50
Octyldodecyl neopentanoate (Elefac 1-205)	5.00
Benzophenone-3	2.50
Minno 21	5.00
Octyl salicylate	5.00
Phase D	
A compound of the invention	0.10

Add phase B to phase A; disperse well and hold at 45°C. Then add phase C slowly. Hold at 45°C, mix well then add phase D. Cool and mix to 30°C and pack.

Example 20: Use of Vinylguaiacol as a functional flavour exhibiting antimicrobial properties

A stock solution of 1g of vinylguaiacol made up to 2ml with de-ionised water was used. This gave a concentration of 10,000 μg/ml vinylguaiacol when 1 ml of the stock solution was added to 49ml of Greek yoghurt (Sainsburys Supermarket). 1 in 4 dilutions were then made up from the remaining stock solution, by taking 1ml of the stock solution and adding this to 1ml of de-ionised water. The same procedure was used for the next solution. This gave the following concentrations of vinylguaiacol in water: 10,000, 2,500, 625, 156.25, 39.06 and 9.77μg/ml. A control was also used: For this 1ml of water was added to 49ml of Greek yoghurt. Each sample was incubated at room temperature, whilst exposed to the air.

After several days, thick confluent growth had occurred in the control. Small colonies were present on the surface of the yoghurts containing the lower concentrations of vinylguaiacol, with the number present gradually decreasing as the concentration of vinylguaiacol used increased until no colonies were present at the highest vinylguaiacol concentrations. Upon further incubation the control developed even thicker growth and sporulation took place, whereas in the presence of vinylguaiacol the colonies barely increased in size and did not increase in number.

Vinylguaiacol appears to exert a bactericidal effect against most types of contaminant. Another useful effect concerned aroma. The control and the yoghurts containing the lowest concentrations of vinylguaiacol had a strong rancid effect, and the yoghurts with the high vinylguaiacol contents had the characteristic clove aroma of vinylguaiacol. However, quite unexpectedly the yoghurts containing the intermediate concentrations of

vinylguaiacol had very little smell, either rancid or of cloves. So it appears that the vinylguaiacol effectively deodorises the rancid smell, perhaps by its antioxidant activity preventing oxidation of milk fats and/or combined with prevention of yoghurt degradation by microbial action.

5

TABLE 15: *Combination of preservative activities*

The inventors have shown that the following compounds of the invention display an unexpected and advantageous combination of preservative activities:

Material	Preservative Activity				
	Photoprotective	Antioxidant	Antimicrobial ❷	Antibrowning	Skin Lightening
Sunflower seed derived					
Protocatechuic acid	-	✓	-	-	✓
Vinylcatechol	-	✓	✓	-	-
Cereal derived					
Vinylguaiacol	-	✓	✓	✓	✓
Methoxyhydroquinone	-	✓	❶	-	-
Ethylguaiacol	-	✓	✓	-	-

❶ Active against bacteria at alkaline pH.

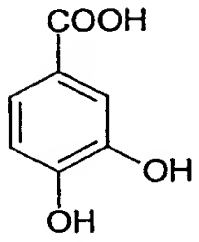
❷ Most of the materials have some antimicrobial activity under some conditions.

Micro-organism Deposits

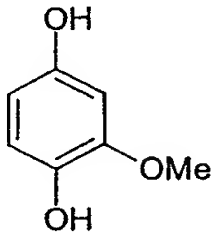
Exemplary micro-organisms suitable for use in accordance with the present invention have been deposited for the purposes of patent procedures under the Budapest Treaty with the IMI Genetic Resource Reference Collection which is an International Depositary authority recognised under the Treaty. The address of the IMI Collection is CABI Bioscience UK Centre Egham, Genetic Resource Collection, Bakeham Lane, Egham, Surrey, England TW20 PTY telephone 01784 470111, fax 01491 829100, e-mail bioscience@cabi.org.

Micro-organisms	Strain Number	Date of acceptance for patent purposes	IMI CC Number
Paecilomyces variotti	ZYL 733	20 November 1998	379901
Rhodotorula glutinis	ZYL 702	20 November 1998	379894
Paenibacillus polymyxa	ZYL 277	24 January 2000	382464
Aspergillus niger	ZYL 768	20 November 1998	379897

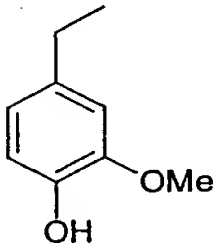
TABLE 16: *Exemplary compounds of the invention*



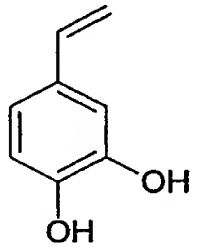
Protocatechuic acid
(3,4-Dihyroxybenzoic acid)



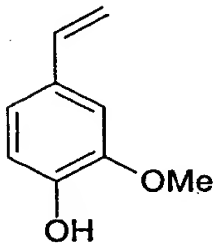
2-Methoxyhydroquinone



Ethylguaiacol
(4-Ethyl-2-methoxyphenol)



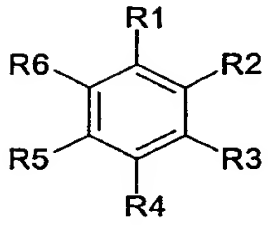
4-Vinylcatechol



4-Vinylguaiacol
(4-Vinyl-2-methoxyphenol)

CLAIMS

1. Use of a compound having the following formula I:



I

wherein R^1 is $CH=CH_2$, COOH, CH_2-CH_3 or OH;
R^3 is OH, or -OCH_3;
R^4 is OH; and
R^2, $R^5$and R^6 are hydrogen,

the compound of formula I optionally being in the form of a salt when it contains a COOH and/or OH group,
as a multifunctional preservative having two or more activities selected from antioxidant, antimicrobial, antibrowning, flavouring/aroma and skin lightener activities.

2. Use as claimed in Claim 1, wherein the compound has antioxidant and antimicrobial activity.

3. Use as claimed in Claim 1, wherein the composition has antioxidant and antibrowning activity.

4. Use as claimed in Claim 1, wherein the composition has flavouring/aroma and antioxidant and/or antimicrobial activity.

5. Use as claimed in Claim 1, wherein the composition has antioxidant and skin lightener activity.

6. Use of a compound as claimed in any one of Claims 1 to 5, wherein the compound is selected from one or more of vinylguaiacol, protocatechuic acid, methoxyhydroquinone and ethylguaiacol.

7. Use of a compound as claimed in any one of Claims 1 to 6, wherein the compound is used in the manufacture of a composition for use as a food, beverage, cosmetic or perfume.

8. Use as claimed in any one of Claims 1 to 7 wherein one or more compounds are provided in the form of an extract derived from a plant material that has been subsequently treated with a microorganism.

9. A composition comprising a compound of formula I, as defined in Claim 1, in combination with an additive which has at least one of the same activities as the compound.

10. A composition comprising two or more different compounds of formula I, as defined in Claim 1.

11. A composition as claimed in Claim 13 further comprising an additive which has at least one of the same activities as the compound.

12. A composition as claimed in any one of Claims 9 to 11, wherein the compound of formula I is in encapsulated form.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 56, line 11

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
IMI GENETIC RESOURCE REFERENCE COLLECTION

Address of depositary institution *(including postal code and country)*

CABI BIOSCIENCE UK CENTRE
BAKEHAM LANE
EGHAM
SURREY TW20 PTY UNITED KINGDOM

Date of deposit	Accession Number
20 November 1998	IMI CC 379901

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC). In respect of the designation of Australia, the expert option is also requested (Regulation 3.25 of Australian Statutory Rules 1991 No. 71). In respect of the designation of Canada, it is similarly requested that only an independent expert nominated by the Commissioner is authorised to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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EGHAM
SURREY TW20 PTY UNITED KINGDOM

Date of deposit	Accession Number
20 November 1998	IMI CC 379894

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CABI BIOSCIENCE UK CENTRE
BAKEHAM LANE
EGHAM
SURREY TW20 PTY UNITED KINGDOM

Date of deposit	Accession Number
24 January 2000	IMI CC 382464

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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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Date of deposit	Accession Number
20 November 1998	IMI CC 379897

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC). In respect of the designation of Australia, the expert option is also requested (Regulation 3.25 of Australian Statutory Rules 1991 No. 71). In respect of the designation of Canada, it is similarly requested that only an independent expert nominated by the Commissioner is authorised to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

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INTERNATIONAL SEARCH REPORT

Int Jonal Application No
PCT/GB 00/00494

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N31/16 A01N37/40 A23L1/226 A23L3/349 A23L3/3508 A61K7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 197727 Derwent Publications Ltd., London, GB; Class D13, AN 1977-47605Y XP002138227 & JP 52 062230 A (AJINOMOTO KK), 23 May 1977 (1977-05-23)	1,4,6-11
Y	abstract	2,3,5,12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2000	06/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lamers, W

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US F.TATEO ET AL.: "Gas chromatographic/mass spectrometry analytical characterization of smoke-liquid flavorings to evaluate the opportunity of their use as antibacterial agents" XP002138221	1,4,6-11
Y	abstract & DEV. FOOD SCI., vol. 37A, 1995, pages 971-979, —	1-3,5,12
X	DATABASE WPI Section Ch, Week 199848 Derwent Publications Ltd., London, GB; Class B05, AN 1998-563047 XP002138228 & JP 10 251136 A (SHISEIDO CO LTD), 22 September 1998 (1998-09-22)	1,3,5-11
Y	abstract —	1,2,4,12
Y	DATABASE WPI Section Ch, Week 198413 Derwent Publications Ltd., London, GB; Class B05, AN 1984-078370 XP002138229 & JP 59 029618 A (HASEGAWA CO LTD), 16 February 1984 (1984-02-16) abstract —	1,2,4
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US M.D.GUILLEN ET AL.: "New Components with Potential Antioxidant and Organoleptic Properties, Detected for the First Time in Liquid Smoke Flavoring Preparations" retrieved from STN-INTERNATIONAL, accession no. 128:243078 CA XP002138222 abstract "IT"	1,3,4, 6-11
Y	& J.AGRIC.FOOD CHEM., vol. 46, no. 4, 1998, pages 1276-1285, — -/--	1,2,5,12

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US A.KOBAYASHI ET AL.: "Uptake and exudation of phenolic compounds by wheat and antimicrobial components of the rot exudate" retrieved from STN-INTERNATIONAL, accession no. PREV199699237302 XP002138223 abstract & ZEITSCHRIFT FÜR NATURFORSCHUNG SECTION C JOURNAL OF BIOSCIENCES, vol. 51, 1996, pages 527-53,	1,2
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X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US C.NERGIZ ET AL.: "Determination of phenolic acids in virgin olive oil" retrieved from STN-INTERNATIONAL, accession no. 114:22632 CA XP002138224 " IT " abstract	1,4,6-11
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Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 152 852 A (PUEHRINGER JOSEF ;MAKES FRANTISEK (SE)) 28 August 1985 (1985-08-28) claim 4	1,2,4
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